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Polyamines are essential for virulence in *Salmonella enterica* serovar Gallinarum despite evolutionary decay of polyamine biosynthesis genes



Casper Schroll^a, Jens P. Christensen^a, Henrik Christensen^a, Susanne E. Pors^a, Lotte Thorndahl^a, Peter R. Jensen^b, John E. Olsen^a, Lotte Jelsbak^{a,*}

^aDepartment of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Denmark

^bSystems Biotechnology, Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark

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ABSTRACT

Serovars of *Salmonella enterica* exhibit different host-specificities where some have broad host-ranges and others, like *S. Gallinarum* and *S. Typhi*, are host-specific for poultry and humans, respectively. With the recent availability of whole genome sequences it has been reported that host-specificity coincides with accumulation of pseudogenes, indicating adaptation of host-restricted serovars to their narrow niches. Polyamines are small cationic amines and in *Salmonella* they can be synthesized through two alternative pathways directly from L-ornithine to putrescine and from L-arginine via agmatine to putrescine. The first pathway is not active in *S. Gallinarum* and *S. Typhi*, and this prompted us to investigate the importance of polyamines for virulence in *S. Gallinarum*. Bioinformatic analysis of all sequenced genomes of *Salmonella* revealed that pseudogene formation of the *speC* gene was exclusive for *S. Typhi* and *S. Gallinarum* and happened through independent events. The remaining polyamine biosynthesis pathway was found to be essential for oral infection with *S. Gallinarum* since single and double mutants in *speB* and *speE*, encoding the pathways from agmatine to putrescine and from putrescine to spermidine, were attenuated. In contrast, *speB* was dispensable after intraperitoneal challenge, suggesting that putrescine was less important for the systemic phase of the disease. In support of this hypothesis, a $\Delta speE;\Delta potCD$ mutant, unable to synthesize and import spermidine, but with retained ability to import and synthesize putrescine, was attenuated after intraperitoneal infection. We therefore conclude that polyamines are essential for virulence of *S. Gallinarum*. Furthermore, our results point to distinct roles for putrescine and spermidine during systemic infection.

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1. Introduction

Salmonella enterica are Gram-negative facultative intracellular bacteria that infect a wide range of animal species as well as humans. The species *S. enterica*

constitutes >2500 different serovars. Depending on the immune status of the infected host and the *Salmonella* serovar, diseases caused by *S. enterica* range from mild diarrhea to life threatening systemic infections. Some serovars like *S. Typhimurium* and *S. Enteritidis* mainly cause a self-limiting diarrhea and are able to cause disease in humans as well as a broad variety of animals. Other serovars like *S. Gallinarum* and *S. Typhi* are host restricted and only cause disease in chickens and humans, respectively. Upon ingestion of *Salmonella* contaminated food or water, the bacterium is able to pass the stomach and reach

* Corresponding author at: Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen, Stigbøjlen 4, DK-1870 Frederiksberg C, Denmark. Tel.: +45 3533 3752; fax: +45 3533 2757.

E-mail address: loj@sund.ku.dk (L. Jelsbak).

the intestines where a specific set of bacterial effector proteins are injected into the epithelial cells lining the wall of the small intestines thereby promoting invasion of these cells (Kaniga et al., 1995a,b). Following invasion of the epithelial cell layer *Salmonella* escapes to the underlying tissues (Knodler et al., 2010) where it is taken up by phagocytes like macrophages and dendritic cells, inside which *Salmonella* is able to survive and replicate, as reviewed in (Mastroeni and Grant, 2011). From here, it can rapidly spread through the lymphoid and blood systems to the spleen and liver resulting in a life-threatening systemic infection. Infections by host-restricted serovars are typically more severe with limited intestinal manifestation, often resulting in life threatening systemic diseases, indicating that host-adaptation has co-evolved with the ability to successfully pass the intestines and cause invasive typhoid-like systemic disease (Uzzau et al., 2001).

Recently, comparative genome analyses have revealed that host-restricted serovars diverged from non-restricted serovars through extensive genome reductions and accumulation of pseudogenes. Specifically, the genomes of *S. Typhi* and *S. Gallinarum* harbor 204 and 309 pseudogenes (Thomson et al., 2008), whereas their closest relatives among the broad host-range serovars *S. Typhimurium* and *S. Enteritidis*, only have 25 and 113 pseudogenes, respectively. This has fostered the hypothesis that genome decay has occurred during pathoadaptive evolution to the specific niches of highly virulent and host-restricted *Salmonella* serovars (Thomson et al., 2008; Holt et al., 2009; Feng et al., 2013). In support of this hypothesis, *S. Gallinarum* and *S. Typhi* have lost several of the same metabolic pathways that are conserved in *S. Typhimurium* and *S. Enteritidis*, suggesting that the host-restricted serovars have similar nutrient resources in their respective intra-host niches (Parkhill et al., 2001; Thomson et al., 2008). As an example, in *S. Typhimurium* putrescine which together with spermidine are the most prominent polyamines in bacteria, can be synthesized via either of two redundant pathways: (1) from L-ornithine by the SpeC or

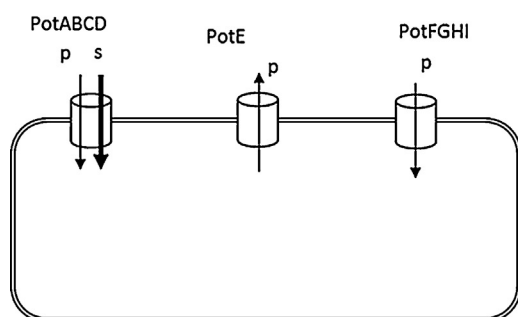
the acid-inducible SpeF ornithine decarboxylase (ODC) or (2) from L-arginine by SpeA and SpeB (Fig. 1). Spermidine is synthesized from putrescine and L-methionine by SpeE and SpeD. In both *S. Gallinarum* and *S. Typhi*, the polyamine biosynthesis gene *speC* encoding an ODC is a pseudogene (Thomson et al., 2008; Holt et al., 2009) and both serovars are classified as ODC negative. The content of polyamines, which represent small poly-cationic molecules present in almost all living cells, is controlled in bacteria by active transport across the membrane and biosynthesis (Igarashi and Kashiwagi, 2009). This indicates that polyamine biosynthesis could be dispensable in host-restricted serovars. As polyamine depletion is deleterious for virulence in *S. Typhimurium* (Jelsbak et al., 2012), these findings prompted us to investigate the role of polyamines in systemic infections of a host-restricted serovar of *S. enterica*. As a model we chose *S. Gallinarum* fowl typhoid infections, a disease highly similar to human typhoid caused by *S. Typhi*. Our results show that polyamines are essential for virulence in *S. Gallinarum*. These results have implications for development of novel treatment regimens for fowl typhoid aimed at inhibiting these enzymes, but also point to a conserved essentiality for polyamines in virulence of both host-restricted and non-restricted serovars of *S. enterica*. Furthermore, both *S. Gallinarum* and *S. Typhi* have lost one of two putrescine biosynthesis pathways supporting the hypothesis that part of the genome reduction observed in host-restricted serovars could be a result of deterioration of redundant metabolic pathways of the notoriously metabolically robust and broad host-range serovars *S. Typhimurium* and *S. Enteritidis*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in Table 1. *S. Gallinarum* G9 was used as wild-type strain in all experiments. This strain has been described previously

A Putrescine/spermidine transport



B Putrescine/spermidine biosynthesis

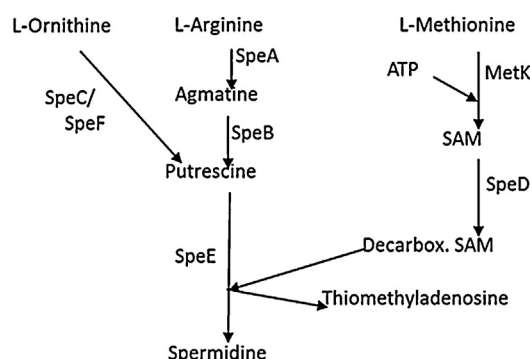


Fig. 1. Graphic presentation of putrescine/spermidine transporters and biosynthesis pathways. (A) A graphic presentation of the polyamine transporters with their substrate affinity indicated by p for putrescine and s for spermidine. (B) A graphic presentation of the polyamine biosynthesis pathways present in bacteria. SAM: S-adenosylmethionine.

Table 1

Strains and plasmid used in the study.

Strains	Relevant genotype	Reference
<i>S. Gallinarum</i> G9	Virulent reference strain	Jones et al. (2001)
CAS87	$\Delta speB$	This work
CAS89	$\Delta speE$	This work
CAS95	$\Delta speB; \Delta speE$	This work
CAS103	$\Delta speB; \Delta speE/pLOJ318$	This work
CAS114	$\Delta potCD$	This work
CAS130	$\Delta potCD; \Delta speE$	This work
CAS159	$\Delta potCD; \Delta speE/pLOJ326$	This work
<i>Plasmids</i>		
pACYC177	Cloning vector, amp ^R , kan ^R	Chang and Cohen (1978)
pKD46	Plasmid with λ -Red recombinase expressed from arabinose inducible promoter	Datsenko and Wanner (2000)
pKD3	Template plasmid for λ -Red mutagenesis, cam ^R	Datsenko and Wanner (2000)
pKD4	Template plasmid for λ -Red mutagenesis, kan ^R	Datsenko and Wanner (2000)
pCP20	FLP plasmid for deletion of resistance cassette	Datsenko and Wanner (2000)
pLOJ318	pACYC177 expressing the <i>speB</i> gene	Jelsbak et al. (2012)
pLOJ326	pACYC177 expressing the <i>potCD</i> genes	Jelsbak et al. (2012)

and its virulence is well defined (Jones et al., 2001; Chadfield et al., 2003). *S. Gallinarum* strains were maintained in LB media (Becton, Dickinson and Company). For solid medium, 1.5% agar was added to give LB agar plates. Chloramphenicol (15 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), ampicillin (100 $\mu\text{g ml}^{-1}$) or carbenicillin (50 $\mu\text{g ml}^{-1}$) was added as required. Prior to all experiments the bacteria were grown for 20 h, 200 rpm, 37 °C in M9 minimal media (2 mM MgSO_4 , 0.1 mM CaCl_2 , 0.4% glucose, 8.5 mM NaCl, 42 mM Na_2HPO_4 , 22 mM KH_2PO_4 , 18.6 mM NH_4Cl) to deplete for carry-over polyamines. To complement for auxotrophy in *S. Gallinarum* G9 the M9 media was supplemented with 0.2 $\mu\text{g ml}^{-1}$ thiamine, 0.2 $\mu\text{g ml}^{-1}$ nicotinic acid, 25 $\mu\text{g ml}^{-1}$ leucine, 5 $\mu\text{g ml}^{-1}$ cystine and 5 $\mu\text{g ml}^{-1}$ aspartic acid.

2.2. Construction of strains

Gene deletions and concomitant insertions of an antibiotic resistance cassette were constructed using Lambda Red mediated recombination as described elsewhere (Datsenko and Wanner, 2000). Double mutants were constructed by P22-mediated transductions after removing the antibiotic resistance cassette in the single mutant by FLP-mediated recombination with introduction of pCP20 (Datsenko and Wanner, 2000). All mutants were verified by PCR. Primers used to construct and verify mutants are listed in Table S1, supplementary material.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2014.01.034>.

2.3. Sequence analysis of polyamine biosynthesis genes

All 33 completed *S. enterica* genomes available in Genbank (Benson et al., 2012) were compared by use of BLAST (Altschul et al., 1997). Sequences of *speC* and *speF* homologs were downloaded and compared by multiple alignment with ClustalX (Larkin et al., 2007). Translation to protein was performed by EMBOSS (Rice et al., 2000).

2.4. Infection of chickens

Chicken challenge experiments were performed by competitive assays, where wild type and mutated strains were inoculated in equal amounts as previously described (Olsen et al., 2013). A few hours after hatching male Lohmann chickens were acclimatized in groups of five animals for three to five days. At all times they were supplied with food and water ad libitum. Three to five days old male Lohmann chickens were challenged in groups of five to ten animals with 10^8 bacteria for oral challenge and 10^6 for intraperitoneal challenge. Competitive indexes (C.I.) were calculated as previously described (Blondel et al., 2010; Jelsbak et al., 2012). The ratio between strains was confirmed by plate count method as described for spleen samples below. After four to five days (within a 24 h interval), the ratio between wild type and mutant strain was determined in the spleen, essentially as described by Jelsbak et al. (2012). In brief, dilutions of homogenized spleens were made in PBS and 100 μl aliquots were plated onto LB agar. *Salmonella* colonies were sub-cultured in parallel onto LB agar with and without the appropriate antibiotics to determine the ratio between wild type and the mutated strains in each animal. A mean competitive index was obtained from each group of individually challenged birds. A C.I. of 1.00 corresponded to wild type virulence. Chicken infection studies were performed with permission from the Danish Animal Experiments Inspectorate, license number 2009/561-1675.

2.5. Infection of HD-11 chicken macrophages

Uptake and survival in the chicken derived macrophage cell line HD-11 was measured using a gentamicin protection assay as previously described using a multiplicity of infection of 10:1 (Wallrodt et al., 2013), the only difference being the exchange of J774 cells with HD-11 cells.

2.6. Statistical analysis

C.I. outliers were identified by Grubbs' test. Comparison of mean ration of input and output pools of wild type and

mutated bacteria in chicken infections were compared by Student *t*-test, and comparisons of CFU in macrophages was performed by ANOVA with Dunn's correction for multiple comparisons. All analysis was performed using GraphPad Prism6.

3. Results and discussion

3.1. *S. Typhi* and *S. Gallinarum* have distinct mutations silencing the *speC* polyamine biosynthesis gene

It has previously been reported that in both *S. Gallinarum* and *S. Typhi* the *speC* gene encoding ornithine decarboxylase is a pseudogene (Parkhill et al., 2001; Thomson et al., 2008), as a result of small out-of-frame deletions leading to premature stop codons. We re-analyzed the sequences and, interestingly, in *S. Gallinarum* there is a 4 base pair deletion after base pair 1268 in the reading frame whereas *S. Typhi* harbors an 11 base pair deletion after base pair 1130 (Table 2). This means that silencing of the *speC* genes in *S. Typhi* and *S. Gallinarum* occurred through independent events and indicates convergent evolution during adaptation to their respective niches, humans and poultry.

In contrast, examination of the *speC* locus of all *Salmonella* genomes in Genbank reveals that in all other serovars than *S. Gallinarum* and *S. Typhi*, the full-length *speC* gene is conserved with only few amino acid differences between strains (Table 2), indicating that silencing of *speC* in *S. Gallinarum* and *S. Typhi* is a relatively recent event occurring after their divergence from their respective ancestors. Interestingly, in *S. Typhi*, the *speF* gene encoding the alternative ODC is also a pseudogene, whereas in *S. Gallinarum*, while the *speF* gene is present, it is not functional as indicated by lack of ODC activity in this bacterium (Crichton and Old, 1990). Most other *Salmonella* strains show positive ODC reactions, indicating that only these highly host-restricted typhoid *Salmonella* strains *S. Gallinarum* and *S. Typhi* have lost the ability to convert ornithine into putrescine,

perhaps reflecting similarities in their respective intra-host environments. Importantly, the alternative biosynthesis pathway to putrescine via *SpeA* and *SpeB* remains intact in all strains analyzed. This is highly indicative of an important role for putrescine biosynthesis in *S. enterica* and is supported by the fact that polyamines are essential for virulence in *S. Typhimurium* (Jelsbak et al., 2012). However, the parallel occurrence of loss-of-function of the ODC-pathway in the typhoid causing host-restricted serovars could reflect limited availability of substrates for putrescine biosynthesis in these serovars.

3.2. Polyamines are essential for virulence in *S. Gallinarum* in orally infected chickens

To analyze the role of polyamines in virulence of the host-restricted serovar, *S. Gallinarum*, individual deletion mutants of *speB* and *speE* were constructed. Deletion of *speB* will abolish putrescine biosynthesis of the strain, as it depends on this pathway for the production of putrescine. The strain is still able to import putrescine from the environment via the PotFGHI transporter. It is likely that reduced putrescine biosynthesis will affect spermidine biosynthesis as well as putrescine is substrate for spermidine biosynthesis by *SpeE*. However, any imported putrescine will be available for conversion to spermidine by *SpeE*. Likewise, deletion of *speE* will abolish spermidine biosynthesis of the strain, as it depends on this pathway for the production of spermidine. This could result in accumulation of putrescine, however, to counter this effect, the strain retains the ability to export excess putrescine via the putrescine exporter PotE. A double mutant of *speB* and *speE* ($\Delta speB;\Delta speE$) was also constructed. This strain is deficient for both putrescine and spermidine biosynthesis, yet retains the ability to take up polyamines from the extracellular environment. Initially, the strains were tested for their ability to grow in minimal media without polyamines (M9). All mutants had growth rates similar to the wt, and when the strains were

Table 2
Comparison of the *SpeC* protein for all published genomes of *Salmonella enterica* subsp. *enterica* serotypes available in Genbank.

SpeC position (Typhimurium)	15	26	40	64	72	79	80	93	105	124	159	272	313	336	378	388	398	423	501	703
Typhimurium	R	D	T	A	V	V	G	C	G	Q	G	S	V	D	N	P	V	R	R	Q
Enteritidis ATCC						G	E	S	D			L								
Enteritidis								S	D			L							Q	
Dublin	C					G		S	D			L					I		Q	
Heidelberg						G														
Javiana			P			G											I		Q	
Schwarzengrund				V	A	G											I		Q	
Choleraesuis						G			D								I		Q	
Paratyphi C						G			D								I		Q	
Weltevreden						G						L					I			
Agona																	I			
Newport						G						L	G	E						
Paratyphi A		E				G				S		P								K
Paratyphi B						G		S	D			L								
Gallinarum						G		S	D			L				L		K ^a		
Typhi		E				G						P			H ^b					

^a From this position out of frame due to 4 bp deletion after bp 1268 resulting in stop codon at position 489.

^b From this position out of frame due to 11 bp deletion after bp 1130 resulting in stop codon at position 418.

Table 3

Competitive index analyses of *S. Gallinarum* mutants in oral infection of chickens.

Mutant vs wt	C.I. \pm S.D.	Statistical analyses
$\Delta speB$	0.14 ± 0.19	Vs 1.0, $p < 0.0001$
$\Delta speE$	0.23 ± 0.2	Vs 1.0, $p = 0.0214$
$\Delta speB; \Delta speE$	0.03 ± 0.03	Vs 1.0, $p < 0.0001$
$\Delta speB; \Delta speE/pACYC-speB$	0.77 ± 1.05	Vs $\Delta speE$, not significant

Infections were performed as described in Section 2. Competitive indexes (C.I.) were calculated as described in Section 2. Standard deviation (S.D.) of the mean is indicated. Statistical analyses were performed as described in Section 2.

incubated for growth overnight they all reached the same stationary level of growth (Supplemental Figure 1).

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2014.01.034>.

Next, the $\Delta speB$ -, $\Delta speE$ -single mutants and the $\Delta speB; \Delta speE$ -double mutants were tested for their ability to pass the intestines and establish a systemic infection in chickens, as measured by bacterial counts in the spleen, after oral inoculations of equal amounts of wt and mutant bacteria as described by Blondel et al. (2010). Both the $\Delta speB$ and the $\Delta speE$ mutant had reduced Competitive indexes (C.I.) of 0.14 ± 0.19 and 0.23 ± 0.2 (Table 3), indicating that both putrescine and spermidine biosynthesis contribute to virulence. The double mutant had an even further reduction in virulence (C.I. = 0.03 ± 0.03) suggesting that polyamine biosynthesis is essential for oral infection of *S. Gallinarum*. Virulence could be partially restored by re-introducing the *speB* gene on a plasmid (C.I. = 0.77 ± 1.05).

3.3. Spermidine is the major contributor to systemic infection of intraperitoneally infected chickens

Passing the intestines and establishing a systemic infection are two discrete steps in the pathogenicity of *Salmonella* serovars (Mastroeni and Grant, 2011). To further elucidate the role of polyamines in the systemic phase of the disease we tested the biosynthesis mutants by intra peritoneal (I.P.) infections of chickens (Table 4). Interestingly, using this route of infection, the $\Delta speB$ -mutant vs the

Table 4

Competitive index analyses of *S. Gallinarum* mutants in intraperitoneal infection.

Mutant vs wt	C.I. \pm S.D.	Statistical analyses
$\Delta speB$	0.98 ± 0.67	Vs 1.0, not significant
$\Delta speE$	0.28 ± 0.08	Vs 1.0, $p < 0.00001$
$\Delta speB; \Delta speE$	0.20 ± 0.06	Vs 1.0, $p < 0.00001$
$\Delta speB; \Delta speE/pACYC-speB$	0.41 ± 0.18	Vs $\Delta speE$, not significant
$\Delta potCD$	0.18 ± 0.21	Vs 1.0, $p = 0.008$
$\Delta potCD; \Delta speE$	0.04 ± 0.04	Vs 1.0, $p < 0.00001$
$\Delta potCD; \Delta speE/pACYC-potCD$	0.21 ± 0.13	Vs $\Delta speE$, not significant

Infections were performed as described in Section 2. Competitive indexes (C.I.) were calculated as described in Section 2. Standard deviation (S.D.) of the mean is indicated. Statistical analyses were performed as described in Section 2.

wt had a C.I. close to 1 (0.98 ± 0.67), indicating that putrescine biosynthesis is less important for the systemic phase of the disease. In contrast, the $\Delta speE$ -mutant had a C.I. of 0.28 ± 0.08 indicating that spermidine biosynthesis does contribute to virulence. The additional deletion of the *speB* gene in this mutant only had a minor effect on the C.I. ($\Delta speB; \Delta speE$ C.I. = 0.2 ± 0.06), supporting the observation that putrescine biosynthesis has a limited role in the systemic phase of the disease.

The $\Delta speE$ -mutant can be expected to be only partially reduced in cellular spermidine concentrations as it retains the ability to take up available spermidine from the host environment through the PotABCD spermidine transporter. We therefore constructed a $\Delta potCD$ -mutant and a $\Delta speE; \Delta potCD$ double mutant to further investigate the specific role of spermidine in virulence. The $\Delta speE; \Delta potCD$ double mutant is unable to synthesize and import spermidine, yet retains the ability to import and synthesize putrescine. The $\Delta potCD$ -mutant had a C.I. of 0.18 ± 0.21 , slightly lower than the $\Delta speE$ -mutant, however the $\Delta speE; \Delta potCD$ double mutant was severely reduced with a C.I. of 0.04 ± 0.04 . Virulence could be restored back to the $\Delta speE$ -mutant level by re-introducing the *potCD* genes on a plasmid. Together, our results strongly indicate that spermidine is essential for systemic infections of chickens by *S. Gallinarum* and that putrescine is less important for the systemic niche. One could argue that attenuation of the *speE*-mutant could be caused by either toxic accumulation of putrescine inside the bacteria, or by reduced access to putrescine in the host environment. However, several of our results argue against these possibilities. Firstly, if putrescine accumulated to toxic levels inside the *speE*-mutant causing its reduced virulence it should not further reduce its virulence by disrupting the spermidine transporter PotABCD. Secondly, the bacteria have a putrescine exporter encoded by the *potE*-gene. This transporter would likely counter the effect of excess intracellular putrescine by pumping it out. Thirdly, if putrescine and spermidine are equally important for systemic infection, we would expect to have a reduced virulence for both the *speB*- and the *speE*-mutants. Rather, the lack of a phenotype for the *speB*-mutant indicates that there is sufficient putrescine in the environment to feed into spermidine biosynthesis by SpeE, but this putrescine does not complement the reduced virulence of the *speE*-mutant. We therefore conclude that spermidine is more important than putrescine for the systemic phase of fowl typhoid.

3.4. Polyamines are not required for intracellular survival and replication of *S. Gallinarum* in cultured HD-11 chicken macrophages

A central feature of *Salmonella* infections is the ability of *Salmonella* to survive and replicate in host cells inside a specialized compartment termed the *Salmonella* containing vacuole (SCV) (Mastroeni and Grant, 2011). To investigate if the reduced virulence of the spermidine mutant was linked to defects in survival inside host cells, intracellular survival and replication of the polyamine mutants was assessed by enumerating intracellular bacteria 2 h, 4 h and 24 h post-infection of HD-11 chicken macrophages. In this assay, all strains had a similar

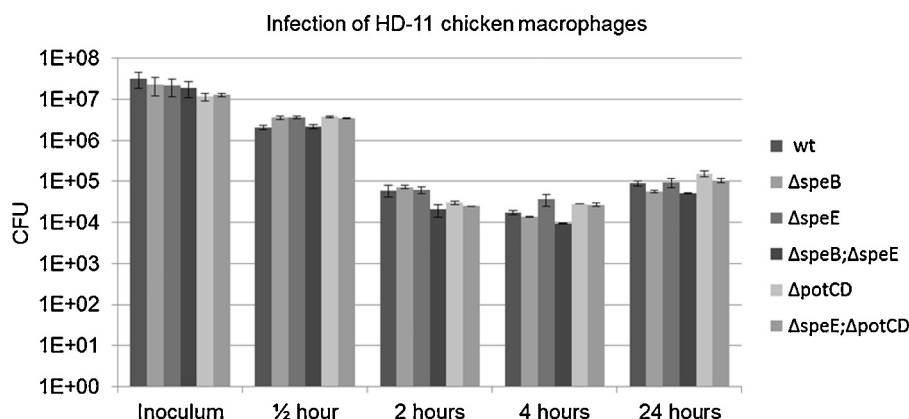


Fig. 2. Intracellular survival of *S. Gallinarum* strains in HD-11 chicken macrophages. HD-11 chicken macrophages were infected with the indicated strains of *S. Gallinarum* as described in Section 2. Bacteria were added to monolayers of HD-11 macrophages and incubated for 30 min. At this point gentamicin was added to the cell-cultures to kill extracellular bacteria. At the indicated time-points, cfu in the wells was measured.

approximate three-to-five fold net-replication between 4 h and 24 h p.i. The level of replication for the wt observed in this assay is similar to the level others have reported (Blondel et al., 2013). In contrast to observation in *S. Typhimurium* (Jelsbak et al., 2012), these results indicate that intracellular replication of *S. Gallinarum* is not affected by polyamines (Fig. 2). The reason for this discrepancy is not known, however, in *S. Typhimurium* intracellular replication was investigated in epithelial cells and only for the first 8 h p.i. perhaps resulting in different results. Alternatively, the specific roles of polyamines in virulence of the two serovars may be diverse, reflecting their diverse disease manifestations in their respective hosts.

4. Concluding remarks

In the present work we show that polyamines are essential for the host-restricted serovar *S. Gallinarum* despite evolutionary loss of some polyamine biosynthesis pathways. These results support the hypothesis that the extensive genome reduction observed in these serovars reflects the narrow niche they occupy where they have lost redundant/excess metabolic pathways compared to the metabolically robust serovars *S. Typhimurium* and *S. Enteritidis* that thrive in diverse environments. Additionally, our results extend recent reports that polyamines are essential for or contribute to virulence of several intracellular pathogens, like *S. Typhimurium*, *Legionella pneumophila*, *Shigella* spp., and *Francisella tularensis* (Carlson, Jr. et al., 2009; Russo et al., 2011; Nasrallah et al., 2011; Barbagallo et al., 2011; Jelsbak et al., 2012). The accumulating evidence on the role of polyamines in virulence of several intracellular pathogens suggests that polyamine biosynthesis could be attractive targets for development of novel anti-infective drugs for treatment of infections by a variety of intracellular bacteria.

It is interesting to note that in *S. Gallinarum* putrescine and spermidine appears to have different roles with spermidine being most important for the systemic phase of the disease. The reasons for this are not clear, however, spermidine has three amine-groups whereas putrescine

only has two. This results in different charges with spermidine having one more positive charge than putrescine thereby affecting the interactions with other molecules of the cell. This could result in distinct physiological roles for the two polyamines. Accordingly, in *Vibrio cholerae*, it has been shown that exogenous spermidine and putrescine have opposite effects on expression of the Type IV pili (Goforth et al., 2013), and in *Bacillus subtilis* only the polyamine norspermidine is a signal for disassembly of biofilms (Kolodkin-Gal et al., 2012). Together with our results, these studies underscore the distinct roles different polyamines may have in bacterial physiology.

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References

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- Barbagallo, M., Di Martino, M.L., Marocchi, L., Pietrangeli, P., De, C.E., Casalino, M., et al., 2011. A new piece of the shigella pathogenicity puzzle: spermidine accumulation by silencing of the *speG* gene. *PLoS ONE* 6, e27226.
- Benson, D.A., Karsch-Mizrachi, I., Clark, K., Lipman, D.J., Ostell, J., Sayers, E.W., 2012. *GenBank*. *Nucleic Acids Res.* 40, D48–D53.
- Blondel, C.J., Yang, H.J., Castro, B., Chiang, S., Toro, C.S., Zaldivar, M., et al., 2010. Contribution of the type VI secretion system encoded in SPI-19 to chicken colonization by *Salmonella enterica* serotypes Gallinarum and Enteritidis. *PLoS ONE* 5, e11724.
- Blondel, C.J., Jimenez, J.C., Leiva, L.E., Alvarez, S.A., Pinto, B.I., Contreras, F., et al., 2013. The type VI secretion system encoded in *Salmonella* pathogenicity island 19 is required for *Salmonella enterica* serotype Gallinarum survival within infected macrophages. *Infect. Immun.* 81, 1207–1220.
- Carlson Jr., P.E., Horzempa, J., O'Dee, D.M., Robinson, C.M., Neophytou, P., Labrinidis, A., Nau, G.J., 2009. Global transcriptional response to spermine, a component of the intramacrophage environment, reveals regulation of *Francisella* gene expression through insertion sequence elements. *J. Bacteriol.* 191, 6855–6864.

- Chadfield, M.S., Brown, D.J., Aabo, S., Christensen, J.P., Olsen, J.E., 2003. Comparison of intestinal invasion and macrophage response of *Salmonella* Gallinarum and other host-adapted *Salmonella enterica* serovars in the avian host. *Vet. Microbiol.* 92, 49–64.
- Chang, A.C., Cohen, S.N., 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* 134, 1141–1156.
- Crichton, P.B., Old, D.C., 1990. *Salmonellae* of serotypes gallinarum and pullorum grouped by biotyping and fimbrial-gene probing. *J. Med. Microbiol.* 32, 145–152.
- Datsenko, K.A., Wanner, B.L., 2000. One step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6640–6645.
- Feng, Y., Johnston, R.N., Liu, G.R., Liu, S.L., 2013. Genomic comparison between *Salmonella* Gallinarum and Pullorum: differential pseudogene formation under common host restriction. *PLOS ONE* 8, e59427.
- Goforth, J.B., Walter, N.E., Karatan, E., 2013. Effects of polyamines on *Vibrio cholerae* virulence properties. *PLoS ONE* 8, e60765.
- Holt, K.E., Thomson, N.R., Wain, J., Langridge, G.C., Hasan, R., Bhutta, Z.A., et al., 2009. Pseudogene accumulation in the evolutionary histories of *Salmonella enterica* serovars Paratyphi A and Typhi. *BMC Genomics* 10, 36.
- Igarashi, K., Kashiwagi, K., 2009. Modulation of cellular function by polyamines. *Int. J. Biochem. Cell Biol.*
- Jelsbak, L., Thomsen, L.E., Wallrodt, I., Jensen, P.R., Olsen, J.E., 2012. Polyamines are required for virulence in *Salmonella enterica* serovar Typhimurium. *PLOS ONE* 7, e36149.
- Jones, M.A., Wigley, P., Page, K.L., Hulme, S.D., Barrow, P.A., 2001. *Salmonella enterica* serovar Gallinarum requires the *Salmonella* pathogenicity island 2 type III secretion system but not the *Salmonella* pathogenicity island 1 type III secretion system for virulence in chickens. *Infect. Immun.* 69, 5471–5476.
- Kaniga, K., Trollinger, D., Galan, J.E., 1995. Identification of two targets of the type III protein secretion system encoded by the *inv* and *spa* loci of *Salmonella* Typhimurium that have homology to the *Shigella* IpaD and IpaA proteins. *J. Bacteriol.* 177, 7078–7085.
- Kaniga, K., Tucker, S., Trollinger, D., Galan, J.E., 1995. Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella* Typhimurium entry into cultured epithelial cells. *J. Bacteriol.* 177, 3965–3971.
- Knodler, L.A., Vallance, B.A., Celli, J., Winfree, S., Hansen, B., Montero, M., Steele-Mortimer, O., 2010. Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. *Proc. Natl. Acad. Sci. U. S. A.*
- Kolodkin-Gal, I., Cao, S., Chai, L., Bottcher, T., Kolter, R., Clardy, J., Losick, R., 2012. A self-produced trigger for biofilm disassembly that targets exopolysaccharide. *Cell* 149, 684–692.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., et al., 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948.
- Mastroeni, P., Grant, A.J., 2011. Spread of *Salmonella enterica* in the body during systemic infection: unravelling host and pathogen determinants. *Expert Rev. Mol. Med.* 13, e12.
- Nasrallah, G.K., Riveroll, A.L., Chong, A., Murray, L.E., Lewis, P.J., Garduno, R.A., 2011. *Legionella pneumophila* requires polyamines for optimal intracellular growth. *J. Bacteriol.* 193, 4346–4360.
- Olsen, J.E., Hoegh-Andersen, K.H., Rosenkrantz, J.T., Schroll, C., Casadesus, J., Aabo, S., Christensen, J.P., 2013. Intestinal invasion of *Salmonella enterica* serovar Typhimurium in the avian host is dose dependent and does not depend on motility and chemotaxis. *Vet. Microbiol.*
- Parkhill, J., Dougan, G., James, K.D., Thomson, N.R., Pickard, D., Wain, J., et al., 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* 413, 848–852.
- Rice, P., Longden, I., Bleasby, A., 2000. EMBOS: the European Molecular Biology Open Software Suite. *Trends Genet.* 16, 276–277.
- Russo, B.C., Horzempa, J., O'Dee, D.M., Schmitt, D.M., Brown, M.J., Carlson Jr., P.E., et al., 2011. A *Francisella tularensis* locus required for spermine responsiveness is necessary for virulence. *Infect. Immun.*
- Thomson, N.R., Clayton, D.J., Windhorst, D., Vernikos, G., Davidson, S., Churcher, C., et al., 2008. Comparative genome analysis of *Salmonella* Enteritidis PT4 and *Salmonella* Gallinarum 287/91 provides insights into evolutionary and host adaptation pathways. *Genome Res.* 18, 1624–1637.
- Uzzau, S., Leori, G.S., Petrucci, V., Watson, P.R., Schianchi, G., Bacciu, D., et al., 2001. *Salmonella enterica* serovar-host specificity does not correlate with the magnitude of intestinal invasion in sheep. *Infect. Immun.* 69, 3092–3099.
- Wallrodt, I., Jelsbak, L., Thorndahl, L., Thomsen, L.E., Lemire, S., Olsen, J.E., 2013. The putative thiosulfate sulfurtransferases PspE and GlpE contribute to virulence of *Salmonella* Typhimurium in the mouse model of systemic disease. *PLoS ONE* 8, e70829.